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(54) Title: HUMAN ENDOKINE ALPHA

(57) Abstract

The present invention concerns a novel member of the tumor necrosis factor (TNF) family of cytokines. In particular, isolated nucleic acid molecules are provided encoding the endokine alpha protein. Endokine alpha polypeptides are also provided, as are vectors, host cells and recombinant methods for producing the same. Also provided are diagnostic and therapeutic methods concerning TNF family-related disorders.

Human Endokine Alpha

Background of the Invention

Field of the Invention

The present invention relates to an endokine alpha protein. In particular, isolated nucleic acid molecules are provided encoding the endokine alpha protein. Endokine alpha polypeptides are also provided, as are vectors, host cells and recombinant methods for producing the same.

Related Art

The cytokine known as tumor necrosis factor-α (TNFα; also termed cachectin) is a protein secreted primarily by monocytes and macrophages in response to endotoxin or other stimuli as a soluble homotrimer of 17 kD protein subunits (Smith, R.A. *et al., J. Biol. Chem. 262*:6951-6954 (1987)). A membrane-bound 26 kD precursor form of TNF has also been described (Kriegler, M. *et al., Cell 53*:45-53 (1988)).

Accumulating evidence indicates that TNF is a regulatory cytokine with pleiotropic biological activities. These activities include: inhibition of lipoprotein lipase synthesis ("cachectin" activity) (Beutler, B. *et al., Nature 316*:552 (1985)), activation of polymorphonuclear leukocytes (Klebanoff, S.J. *et al., J. Immunol. 136*:4220 (1986); Perussia, B., *et al., J. Immunol. 138*:765 (1987)), inhibition of cell growth or stimulation of cell growth (Vilcek, J. *et al., J. Exp. Med. 163*:632 (1986); Sugarman, B.J. *et al., Science 230*:943 (1985); Lachman, L.B. *et al., J. Immunol. 138*:2913 (1987)), cytotoxic action on certain transformed cell types (Lachman, L.B. *et al.*, supra; Darzynkiewicz, Z. *et al., Canc. Res. 44*:83 (1984)), antiviral activity (Kohase, M. *et al., Cell 45*:659 (1986); Wong, G.H.W. *et al., Nature 323*:819 (1986)), stimulation of bone resorption (Bertolini, D.R. *et al., Nature 319*:516 (1986); Saklatvala, J., *Nature 322*:547 (1986)), stimulation of collagenase and prostaglandin E2 production (Dayer, J.-M. *et al., J. Exp. Med.*

Forum, p.463-466 (1989); Simpson, S.Q. *et al.*, *Crit. Care Clin. 5*:27-47 (1989)), including fever, malaise, anorexia, and cachexia. Endotoxin is a potent monocyte/macrophage activator which stimulates production and secretion of TNF (Kornbluth, S.K. *et al.*, *J. Immunol. 137*:2585-2591 (1986)) and other cytokines. Because TNF could mimic many biological effects of endotoxin, it was concluded to be a central mediator responsible for the clinical manifestations of endotoxin-related illness. TNF and other monocyte-derived cytokines mediate the metabolic and neurohormonal responses to endotoxin (Michie, H.R. *et al.*, *N. Eng. J. Med. 318*:1481-1486 (1988)). Endotoxin administration to human volunteers produces acute illness with flu-like symptoms including fever, tachycardia, increased metabolic rate and stress hormone release (Revhaug, A. *et al.*, *Arch. Surg. 123*:162-170 (1988)). Elevated levels of circulating TNF have also been found in patients suffering from Gram-negative sepsis (Waage, A. *et al.*, *Lancet 1*:355-357 (1987); Hammerle, A.F. *et al.*, *Second Vienna Shock Forum* p. 715-718 (1989); Debets, J.M.H. *et al.*, *Crit. Care Med. 17*:489-497 (1989); Calandra, T. *et al.*, *J. Infec. Dis. 161*:982-987 (1990)).

Passive immunotherapy directed at neutralizing TNF may have a beneficial effect in Gram-negative sepsis and endotoxemia, based on the increased TNF production and elevated TNF levels in these pathology states, as discussed above.

Antibodies to a "modulator" material which was characterized as cachectin (later found to be identical to TNF) were disclosed by Cerami *et al.* (EPO Patent Publication 0,212,489, March 4, 1987). Such antibodies were said to be useful in diagnostic immunoassays and in therapy of shock in bacterial infections. Rubin *et al.* (EPO Patent Publication 0,218,868, April 22, 1987) disclosed monoclonal antibodies to human TNF, the hybridomas secreting such antibodies, methods of producing such antibodies, and the use of such antibodies in immunoassay of TNF. Yone *et al.* (EPO Patent Publication 0,288,088, October 26, 198°) disclosed anti-TNF antibodies, including mAbs, and their utility in immunoassay diagnosis of pathologies, in particular Kawasaki's pathology and bacterial infection. The body fluids of patients with Kawasaki's

Sequence analysis of cytokine receptors has defined several subfamilies of membrane proteins (1) the Ig superfamily, (2) the hematopoietin (cytokine receptor superfamily and (3) the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily (for review of TNF superfamily see, Gruss and Dower, *Blood 85(12)*:3378-3404 (1995) and Aggarwal and Natarajan, *Eur. Cytokine Netw., 7(2)*:93-124 (1996)). The TNF/NGF receptor superfamily contains at least 10 difference proteins. Gruss and Dower, *supra*. Ligands for these receptors have been identified and belong to at least two cytokine superfamilies. Gruss and Dower, *supra*.

Accordingly, there is a need to provide cytokines similar to TNF that are involved in pathological conditions. Such novel cytokines could be used to make novel antibodies or other antagonists that bind these TNF-like cytokines for therapy of TNF-like disorders.

Summary of the Invention

The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding a cytokine that is similar to TNF and is believed to have similar biological effects and activities. This cytokine is named endokine alpha, and includes endokine alpha polypeptides having at least a portion of the amino acid sequence in FIG. 1 (SEQ ID NO:2) or an amino acid sequence encoded by the cDNA clone deposited in a bacterial host as ATCC Deposit Number 97640 on June 27, 1996. The nucleotide sequence, which was determined by sequencing the deposited endokine alpha cDNA clone, contains an open reading frame encoding a polypeptide of about 169 amino acid residues including an N-terminal methionine, an intracellular domain of about 17 amino acid residues, a transmembrane domain of about 26 amino acids, an extracellular domain of about 126 amino acids, and a deduced molecular weight for the complete protein of about 19 kDa. The 126 amino acid sequence of the expected mature endokine alpha protein is shown in FIG. 1 (SEQ ID NO:2) (residues 44 to 169).

Preferred polypeptide fragments according to the present invention include a polypeptide comprising: the endokine alpha intracellular domain, the endokine alpha transmembrane domain, and the endokine alpha extracellular domain.

The invention further provides methods for isolating antibodies that bind specifically to an endokine alpha polypeptide having an amino acid sequence as described above. Such antibodies may be useful diagnostically or therapeutically as antagonists in the treatment of endokine alpha- and/or TNF-related disorders.

Brief Description of the Figures

FIG. 1 shows the nucleotide (SEQ ID NO:1) and deduced amino acid (SEQ ID NO:2) sequences of the endokine alpha protein. Amino acids 1 to 17 represent the intracellular domain, amino acids 18 to 43 the transmembrane domain (the underlined sequence), and amino acids 44 to 169 the extracellular domain (the remaining sequence).

FIG. 2 shows the regions of similarity between the amino acid sequences of the endokine alpha protein (SEQ ID NO:2), tissue necrosis factor α (TNF-α) (SEQ ID NO:3), and TNF-β (SEQ ID NO:4). The J. Hein method was used with PAM 250 residue weight table. Shading with solid black indicates residues that match consensus exactly.

FIG. 3 provides an analysis of the endokine alpha amino acid sequence. Alpha, beta, turn and coil regions; hydrophilicity and hydrophobicity; amphipathic regions; flexible regions; antigenic index and surface probability are shown. In the "Antigenic Index - Jameson-Wolf" graph, amino acid residues 44-54, 57-68, 69-78, 94-105, 108-132 and 148-158 in FIG. 1 correspond to the shown highly antigenic regions of the endokine alpha protein.

encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

Unless otherwise indicated, each "nucleotide sequence" set forth herein is presented as a sequence of deoxyribonucleotides (abbreviated A, G , C and T). However, by "nucleotide sequence" of a nucleic acid molecule or polynucleotide is intended, for a DNA molecule or polynucleotide, a sequence of deoxyribonucleotides, and for an RNA molecule or polynucleotide, the corresponding sequence of ribonucleotides (A, G, C and U) where each thymidine deoxynucleotide (T) in the specified deoxynucleotide sequence in is replaced by the ribonucleotide uridine (U). For instance, reference to an RNA molecule having the sequence of FIG. 1 (SEQ ID NO:1) set forth using deoxyribonucleotide abbreviations is intended to indicate an RNA molecule having a sequence in which each deoxynucleotide A, G or C of SEQ ID NO:1 has been replaced by the corresponding ribonucleotide A, G or C, and each deoxynucleotide T has been replaced by a ribonucleotide U.

Using the information provided herein, such as the nucleotide sequence in FIG. 1, a nucleic acid molecule of the present invention encoding an endokine alpha polypeptide can be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA as starting material. Illustrative of the invention, the nucleic acid molecule described in FIG. 1 (SEQ ID NO:1) was discovered in a cDNA library derived from human brain striatum. Expressed sequence tags corresponding to a portion of the endokine alpha cDNA were also found in several endothelial libraries and a fetal liver library.

The endokine alpha gene contains an open reading frame encoding a protein of about 169 amino acid residues, an intracellular domain of about 17 amino acids (amino acid residues from about 1 to about 17 in FIG. 1 (SEQ ID NO:2)), a transmembrane domain of about 26 amino acids (amino acid residues from about 18 to about 43 in FIG. 1 (SEQ ID NO:2)). an extracellular domain of about 126 amino acids (amino acid residues from about 44 to about 169 in FIG. 1 (SEQ ID NO:2)); and a deduced molecular weight of about 19 kDa. The

Isolated nucleic acid molecules of the present invention include DNA molecules comprising the open reading frame (ORF) shown in FIG. 1 (SEQ ID NO:1) and further include nucleic acid molecules substantially different than all or part of the ORF sequence shown in FIG. 1(SEQ ID NO:1) but which, due to the degeneracy of the genetic code, still encode the endokine alpha protein or a fragment thereof. Of course, the genetic code is well known in the art. Thus, it would be routine for one skilled in the art to generate the degenerate variants described above.

In another aspect, the invention provides isolated nucleic acid molecules encoding the endokine alpha polypeptide having an amino acid sequence encoded by the cDNA of the clone deposited as ATCC Deposit No. 97640 on June 27, 1996. Preferably, the nucleic acid molecule will encode the mature polypeptide encoded by the above-described deposited cDNA clone. The invention further provides an isolated nucleic acid molecule having the nucleotide sequence shown in FIG. 1 (SEQ ID NO:1) or the nucleotide sequence of the endokine alpha cDNA contained in the above-described deposited clone, or a nucleic acid molecule having a sequence complementary to one of the above sequences. Such isolated molecules, particularly DNA molecules, are useful as probes for gene mapping by *in situ* hybridization with chromosomes and for detecting expression of the endokine alpha gene in human tissue, for instance, by Northern blot analysis. As described in detail below, detecting altered endokine alpha gene expression in certain tissues or bodily fluids is indicative of certain disorders.

The present invention is further directed to fragments of the isolated nucleic acid molecules described herein. By a fragment of an isolated nucleic acid molecule having the nucleotide sequence of the deposited cDNA or the nucleotide sequence shown in FIG. 1 (SEQ ID NO. 1) is intended fragments at least about 15 nt, and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably, at least about 40 nt in length which are useful as diagnostic probes and primers as discussed herein. Of course, larger fragments 50-1500 nt in length are also useful according to the present invention as are fragments corresponding to most, if not all, of the nucleotide sequence of

Methods for determining other such epitope-bearing portions of the endokine alpha protein are described in detail below.

In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, the cDNA clone contained in ATCC Deposit 97640 made on June 27, 1996. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C. By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. These are useful as diagnostic probes and primers as discussed above and in more detail below.

Of course, polynucleotides hybridizing to a larger portion of the reference polynucleotide (*e.g.*, the deposited cDNA clone), for instance, a portion 50-500 nt in length, or even to the entire length of the reference polynucleotide, are also useful as probes according to the present invention, as are polynucleotides corresponding to most, if not all, of the nucleotide sequence of the deposited cDNA or the nucleotide sequence as shown in FIG. 1 (SEQ ID NO:1). By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide, (*e.g.*, the deposited cDNA or the nucleotide sequence as shown in FIG. 1 (SEQ ID NO:1)). As indicated, such portions are useful diagnostically either as a probe according to conventional DNA hybridization techniques or as primers for amplification of a target sequence by the polymerase chain reaction (PCR), as described, for instance, in Sambrook, J. *et al.*, eds., *Molecular Cloning, A Laboratory Manual*, 2nd. edition, Cold Spring Harbor Laboratory Press, Cold

encoding a peptide which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are publicly and/or commercially available. As described in Gentz *et al., Proc. Natl. Acad. Sci. USA 86*:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The "HA" tag is another peptide useful for purification which corresponds to an epitope derived from the influenza hemagglutinin (HA) protein, which has been described by Wilson *et al., Cell 37*:767 (1984). Other such fusion proteins include the endokine alpha protein fused to Fc at the N- or C-terminus.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the endokine alpha protein. Variants can occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. Non-naturally occurring variants can be produced, *e.g.*, using art-known mutagenesis techniques.

Such variants include those produced by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. The variants can be altered in coding or non-coding regions or both. Alterations in the coding regions can produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the endokine alpha protein or portions thereof. Also especially preferred in this regard are conservative substitutions. Most highly preferred are nucleic acid molecules encoding the mature endokine alpha protein having the amino acid sequence shown in FIG. 1 (SEQ ID NO:2) or the mature endokine alpha amino acid sequence encoded by the deposited cDNA clone.

among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 90, 95%, 96%, 97%, 98%, or 99% identical to, for instance, the nucleotide sequence shown in FIG. 1 or to the nucleotides sequence of the deposited cDNA clone can be determined conventionally using known computer programs such as the BESTFIT program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). BESTFIT uses the local homology algorithm of Smith and Waterman, *Adv. Appl. Math. 2*:482-489 (1981), to find the best segment of homology between two sequences. When using BESTFIT or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

The present application is directed to such nucleic acid molecules which are at least 90%, 95%, 96%, 97%, 98%, or 99% identical to a nucleic acid sequence described above irrespective of whether they encode a polypeptide having endokine alpha protein activity. This is because, even where a particular nucleic acid molecule does not encode a polypeptide having endokine alpha activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having endokine alpha activity include, *inter alia*, (1) isolating the endokine alpha gene or allelic variants thereof from a cDNA library; (2) *in situ* hybridization (FISH) to metaphase chromosomal spreads to provide precise chromosomal location of the endokine alpha gene as described in Verma *et al.*, *Human Chromosomes: a Manual of Basic Techniques*, Pergamon

rejected by natural selection. The second approach uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene and selections or screens to identify sequences that maintain functionality. As the authors state, these studies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which amino acid changes are likely to be permissive at a certain position of the protein. For example, most buried amino acid residues require nonpolar side chains, whereas few features of surface side chains are generally conserved. Other such phenotypically silent substitutions are described in Bowie, J.U., *et al., supra,* and the references cited therein.

Vectors and Host Cells

The present invention also relates to vectors which include the isolated DNA molecules of the present invention, host cells which are genetically engineered with the recombinant vectors, and the production of endokine alpha polypeptides or portions thereof by recombinant techniques.

Recombinant constructs may be introduced into host cells using well known techniques such as infection, transduction, transfection, transvection, electroporation and transformation. The vector may be, for example, a phage, plasmid, viral or retroviral vector. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells.

The polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged *in vitro* using an appropriate packaging cell line and then transduced into host cells.

Preferred are vectors comprising cis-acting control regions to the polynucleotide of interest. Appropriate trans-acting factors may be supplied by

Among vectors preferred for use in bacteria include pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

Among known bacterial promoters suitable for use in the present invention include the *E. coli lacI* and *lacZ* promoters, the T3 and T7 promoters, the *gpt* promoter, the lambda PR and PL promoters and the *trp* promoter. Suitable eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus (RSV), and metallothionein promoters, such as the mouse metallothionein-I promoter.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al.*, *Basic Methods in Molecular Biology* (1986).

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act to increase transcriptional activity of a promoter in a given host cell-type. Examples of enhancers include the SV40 enhancer, which is located on the late side of the replication origin at bp 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the expressed polypeptide.

or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

Endokine Alpha Polypeptides and Peptides

The invention further provides an isolated endokine alpha polypeptide having the amino acid sequence encoded by the deposited cDNA, or the amino acid sequence in FIG. 1 (SEQ ID NO:2), or a peptide or polypeptide comprising a portion of the above polypeptides. The terms "peptide" and "oligopeptide" are considered synonymous (as is commonly recognized) and each term can be used interchangeably as the context requires to indicate a chain of at least two amino acids coupled by peptidyl linkages. The word "polypeptide" is used herein for chains containing more than ten amino acid residues. All oligopeptide and polypeptide formulas or sequences herein are written from left to right and in the direction from amino terminus to carboxy terminus.

By "isolated" polypeptide or protein is intended a polypeptide or protein removed from its native environment. For example, recombinantly produced polypeptides and proteins expressed in recombinant host cells are considered isolated for purposes of the invention as are native or recombinant polypeptides and proteins which have been substantially purified by any suitable technique

transmembrane domain, and the extracellular domain, as well as polypeptides which have at least 90% similarity, more preferably at least 95% similarity, and still more preferably at least 96%, 97%, 98% or 99% similarity to a polypeptide described herein. Further polypeptides of the present invention include polypeptides at least 80% identical, more preferably at least 90% or 95% identical, still more preferably at least 96%, 97%, 98% or 99% identical to a polypeptide described herein, and also include portions of such polypeptides with at least 30 amino acids and more preferably at least 50 amino acids.

By "% similarity" for two polypeptides is intended a similarity score produced by comparing the amino acid sequences of the two polypeptides using the BESTFIT program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711) and the default settings for determining similarity. BESTFIT uses the local homology algorithm of Smith and Waterman, *Adv. Appl. Math. 2*:482-489 (1981), to find the best segment of similarity between two sequences.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of an endokine alpha polypeptide is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid sequence of the endokine alpha polypeptide. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

epitopes. See, for instance, Geysen, H.M. *et al.*, *Proc. Natl. Acad. Sci. USA 81*:3998-4002 (1984).

As to the selection of peptides or polypeptides bearing an antigenic epitope (i.e., that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. *See*, for instance, Sutcliffe, J.G. *et al.*, *Science 219*:660-666 (1983). Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (*i.e.*, immunogenic epitopes) nor to the amino or carboxyl terminals. Peptides that are extremely hydrophobic and those of six or fewer residues generally are ineffective at inducing antibodies that bind to the mimicked protein; longer, soluble peptides, especially those containing proline residues, usually are effective. Sutcliffe *et al.*, *supra*, at 661. For instance, 18 of 30 peptides designed according to these guidelines, containing 8-39 residues covering 75% of the sequence of the influenza virus hemagglutinin HA1 polypeptide chain, induced antibodies that reacted with the HA1 protein or intact virus; and 12/12 peptides from the MuLV polymerase and 18/18 from the rabies glycoprotein induced antibodies that precipitated the respective proteins.

Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. Thus, a high proportion of hybridomas obtained by fusion of spleen cells from donors immunized with an antigen epitope-bearing peptide generally secrete antibody reactive with the native protein. Sutcliffe *et al.*, *supra*, at 663. The antibodies raised by antigenic epitope-bearing peptides or polypeptides are useful to detect the mimicked protein, and antibodies to different peptides may be used for tracking the fate of various regions of a protein precursor which undergoes posttranslational processing. The peptides and anti-peptide antibodies may be used in a variety of qualitative or quantitative assays for the mimicked protein, for instance in

indicated above, the inventors have determined that the above polypeptide fragments are antigenic regions of the endokine alpha protein.

The epitope-bearing peptides and polypeptides of the invention may be produced by any conventional means for making peptides or polypeptides including recombinant means using nucleic acid molecules of the invention. For instance, a short epitope-bearing amino acid sequence may be fused to a larger polypeptide which acts as a carrier during recombinant production and purification, as well as during immunization to produce anti-peptide antibodies. Epitope-bearing peptides also may be synthesized using known methods of chemical synthesis. For instance, Houghten has described a simple method for synthesis of large numbers of peptides, such as 10-20 mg of 248 different 13 residue peptides representing single amino acid variants of a segment of the HA1 polypeptide which were prepared and characterized (by ELISA-type binding studies) in less than four weeks. *See*, Houghten, R.A., *Proc. Natl. Acad. Sci. USA 82*:5131-5135 (1985). This "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten *et al.* (1986). In this procedure the individual resins for the solid-phase synthesis of various peptides are contained in separate solvent-permeable packets, enabling the optimal use of the many identical repetitive steps involved in solid-phase methods. A completely manual procedure allows 500-1000 or more syntheses to be conducted simultaneously. Houghten *et al.*, *supra*, at 5134.

Epitope-bearing peptides and polypeptides of the invention are used to induce antibodies according to methods well known in the art. See, for instance, Sutcliffe *et al.*, *supra*; Wilson *et al.*, *supra*; Chow, M. *et al.*, *Proc. Natl. Acad. Sci. USA 82*:910-914; and Bittle, F.J. *et al.*, *J. Gen. Virol. 66*:2347-2354 (1985). Generally, animals may be immunized with free peptide; however, anti-peptide antibody titer may be boosted by coupling of the peptide to a macromolecular carrier, such as keyhole limpet hemocyanin (KLH) or tetanus toxoid. For instance, peptides containing cysteine may be coupled to carrier using a linker such as m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), while other

Geysen (1987) further describes this method of identifying a peptide bearing an immunogenic epitope of a desired protein.

Further still, U.S. Patent No. 5,194,392 to Geysen (1990) describes a general method of detecting or determining the sequence of monomers (amino acids or other compounds) which is a topological equivalent of the epitope (i.e., a "mimotope") which is complementary to a particular paratope (antigen binding site) of an antibody of interest. More generally, U.S. Patent No. 4,433,092 to Geysen (1989) describes a method of detecting or determining a sequence of monomers which is a topographical equivalent of a ligand which is complementary to the ligand binding site of a particular receptor of interest. Similarly, U.S. Patent No. 5,480,971 to Houghten, R. A. *et al.* (1996) on Peralkylated Oligopeptide Mixtures discloses linear C_1-C_7-alkyl peralkylated oligopeptides and sets and libraries of such peptides, as well as methods for using such oligopeptide sets and libraries for determining the sequence of a peralkylated oligopeptide that preferentially binds to an acceptor molecule of interest. Thus, non-peptide analogs of the epitope-bearing peptides of the invention also can be made routinely by these methods.

The present inventors have discovered that the endokine alpha protein is a 169 residue protein exhibiting three main structural domains. The intracellular domain was identified within residues from about 1 to about 17 in FIG. 1 (SEQ ID NO:2). The transmembrane domain was identified within residues from about 18 to about 43 in FIG. 1 (SEQ ID NO:2). The extracellular domain was identified within residues from about 44 to about 169 in FIG. 1 (SEQ ID NO:2). Thus, the invention further provides preferred endokine alpha protein fragments comprising a polypeptide selected from: the endokine alpha intracellular domain, the transmembrane domain and the endokine alpha extracellular domain.

The extracellular domain of the endokine alpha protein can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric polypeptides. These fusion proteins show an increased half-life *in vivo*. This has been shown, e.g., for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant

biological sample). Preferably, the endokine alpha protein level or mRNA level in the first biological sample is measured or estimated and compared to a standard endokine alpha protein level or mRNA level, the standard being taken from a second biological sample obtained from an individual not having the disorder or being determined by averaging levels from a population of individuals not having a disorder involving endokine alpha. As will be appreciated in the art, once a standard endokine alpha protein level or mRNA level is known, it can be used repeatedly as a standard for comparison.

By "biological sample" is intended any biological sample obtained from an individual, body fluid, cell line, tissue culture, or other source which contains endokine alpha protein or mRNA. As indicated, biological samples include body fluids (such as sera, plasma, urine, synovial fluid and spinal fluid) which contain secreted mature endokine alpha protein, or tissue sources found to express endokine alpha. Methods for obtaining tissue biopsies and body fluids from mammals are well known in the art. Where the biological sample is to include mRNA, a tissue biopsy is the preferred source.

The present invention is useful for diagnosis of various endokine alpha - related disorders in mammals, preferably humans, as similar to TNF-like disorders known in the art or as presented herein. These include disorders associated with immunomodulation and inflammation, cell proliferation, angiogenesis, tumor metastases, apoptosis, sepsis and endotoxemia.

Total cellular RNA can be isolated from a biological sample using any suitable technique such as the single-step guanidinium-thiocyanate-phenol-chloroform method described in Chomczynski and Sacchi, *Anal. Biochem. 162*:156-159 (1987). Levels of mRNA encoding an endokine alpha polypeptide are then assayed using any appropriate method. These include Northern blot analysis, S1 nuclease mapping, the polymerase chain reaction (PCR), reverse transcription in combination with the polymerase chain reaction (RT-PCR), and reverse transcription in combination with the ligase chain reaction (RT-LCR).

Northern blot analysis can be performed as described in Harada *et al.*, *Cell 63*:303-312 (1990). Briefly, total RNA is prepared from a biological sample as

conventional techniques. After a suitable number of rounds to achieve amplification, the PCR reaction mixture is electrophoresed on a polyacrylamide gel. After drying the gel, the radioactivity of the appropriate bands (corresponding to the mRNA encoding the endokine alpha protein) is quantified using an imaging analyzer. RT and PCR reaction ingredients and conditions, reagent and gel concentrations, and labeling methods are well known in the art. Variations on the RT-PCR method will be apparent to the skilled artisan.

Any set of oligonucleotide primers which will amplify reverse transcribed target mRNA can be used and can be designed as described in the sections above.

Assaying endokine alpha protein levels in a biological sample can occur using any art-known method. Preferred for assaying endokine alpha protein levels in a biological sample are antibody-based techniques. For example, endokine alpha protein expression in tissues can be studied with classical immunohistological methods. In these, the specific recognition is provided by the primary antibody (polyclonal or monoclonal) but the secondary detection system can utilize fluorescent, enzyme, or other conjugated secondary antibodies. As a result, an immunohistological staining of tissue section for pathological examination is obtained. Tissues can also be extracted, e.g., with urea and neutral detergent, for the liberation of endokine alpha protein for Western-blot or dot/slot assay (Jalkanen, M., *et al., J. Cell. Biol. 101*:976-985 (1985); Jalkanen, M., *et al., J. Cell . Biol. 105*:3087-3096 (1987)). In this technique, which is based on the use of cationic solid phases, quantitation of endokine alpha protein can be accomplished using isolated endokine alpha protein as a standard. This technique can also be applied to body fluids. With these samples, a molar concentration of endokine alpha protein will aid to set standard values of endokine alpha protein content for different body fluids, like serum, plasma, urine, synovial fluid, spinal fluid, etc. The normal appearance of endokine alpha protein amounts can then be set using values from healthy individuals, which can be compared to those obtained from a test subject.

Other antibody-based methods useful for detecting endokine alpha protein levels include immunoassays, such as the enzyme linked immunosorbent assay

alpha protein include those detectable by X-radiography, NMR or ESR. For X-radiography, suitable labels include radioisotopes such as barium or cesium, which emit detectable radiation but are not overtly harmful to the subject. Suitable markers for NMR and ESR include those with a detectable characteristic spin, such as deuterium, which may be incorporated into the antibody by labeling of nutrients for the relevant hybridoma.

A endokine alpha protein-specific antibody or antibody portion which has been labeled with an appropriate detectable imaging moiety, such as a radioisotope (for example, ^{131}I, ^{111}In, ^{99m}Tc), a radio-opaque substance, or a material detectable by nuclear magnetic resonance, is introduced (for example, parenterally, subcutaneously or intraperitoneally) into the mammal to be examined for a disorder. It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moieties needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of ^{99m}Tc. The labeled antibody or antibody portion will then preferentially accumulate at the location of cells which contain endokine alpha protein. *In vivo* tumor imaging is described in S. W. Burchiel *et al.*, "Immunopharmacokinetics of Radiolabeled Antibodies and Their Portions" (Chapter 13 in *Tumor Imaging: The Radiochemical Detection of Cancer*, Burchiel, S.W. and Rhodes, B.A. eds., Masson Publishing Inc. (1982)).

Endokine alpha-protein specific antibodies for use in the present invention can be raised against the intact endokine alpha protein or an antigenic polypeptide portion thereof, which may presented together with a carrier protein, such as an albumin, to an animal system (such as rabbit or mouse) or, if it is long enough (at least about 25 amino acids), without a carrier.

As used herein, the term "antibody" (Ab) or "monoclonal antibody" (Mab) is meant to include intact molecules as well as antibody portions (such as, for example, Fab and $F(ab')_2$ portions) which are capable of specifically binding to endokine alpha protein. Fab and $F(ab')_2$ portions lack the Fc portion of intact antibody, clear more rapidly from the circulation, and may have less non-specific

(1981); Harlow & Lane, *infra*, Chapter 7. The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding the endokine alpha antigen.

Alternatively, additional antibodies capable of binding to the endokine alpha protein antigen may be produced in a two-step procedure through the use of anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and therefore it is possible to obtain an antibody which binds to a second antibody. In accordance with this method, endokine alpha protein specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an animal are then used to produce hybridoma cells, and the hybridoma cells are screened to identify clones which produce an antibody whose ability to bind to the endokine alpha protein-specific antibody can be blocked by the endokine alpha protein antigen. Such antibodies comprise anti-idiotypic antibodies to the endokine alpha protein-specific antibody and can be used to immunize an animal to induce formation of further endokine alpha protein-specific antibodies.

It will be appreciated that Fab and $F(ab')_2$ and other portions of the antibodies of the present invention may be used according to the methods disclosed herein. Such portions are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab portions) or pepsin (to produce $F(ab')_2$ portions). Alternatively, endokine alpha protein-binding portions can be produced through the application of recombinant DNA technology or through synthetic chemistry.

Where *in vivo* imaging is used to detect enhanced levels of endokine alpha protein for diagnosis in humans, it may be preferable to use "humanized" chimeric monoclonal antibodies. Such antibodies can be produced using genetic constructs derived from hybridoma cells producing the monoclonal antibodies described above. Methods for producing chimeric antibodies are known in the art. See, for review, Morrison, *Science* 229:1202 (1985); Oi *et al.*, *BioTechniques* 4:214 (1986); Cabilly *et al.*, U.S. Patent No. 4,816,567; Taniguchi *et al.*, EP 171496; Morrison *et al.*, EP 173494; Neuberger *et al.*, WO 8601533; Robinson

acridinium salt label, an oxalate ester label, a luciferin label, a luciferase label, and an aequorin label.

Examples of nuclear magnetic resonance contrasting agents include heavy metal nuclei such as Gd, Mn, and Fe.

Typical techniques for binding the above-described labels to antibodies are provided by Kennedy *et al.* (*Clin. Chim. Acta 70*:1-31 (1976)), and Schurs *et al.* (*Clin. Chim. Acta 81*:1-40 (1977)). Coupling techniques mentioned in the latter are the glutaraldehyde method, the periodate method, the dimaleimide method, the m-maleimidobenzyl-N-hydroxy-succinimide ester method, all of which methods are incorporated by reference herein.

Chromosome Assays

The nucleic acid molecules of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

In certain preferred embodiments in this regard, the cDNA herein disclosed is used to clone genomic DNA of an endokine alpha protein gene. This can be accomplished using a variety of well known techniques and libraries, which generally are available commercially. The genomic DNA then is used for *in situ* chromosome mapping using well known techniques for this purpose. Typically, in accordance with routine procedures for chromosome mapping, some trial and error may be necessary to identify a genomic probe that gives a good *in situ* hybridization signal.

in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

Endokine Alpha Protein and Antibody Therapy

As indicated above, TNF is noted for its pro-inflammatory actions which result in tissue injury, such as induction of procoagulant activity on vascular endothelial cells (Pober, J.S. *et al., J. Immunol. 136*:1680 (1986)), increased adherence of neutrophils and lymphocytes (Pober, J.S. *et al., J. Immunol. 138*:3319 (1987)), and stimulation of the release of platelet activating factor from macrophages, neutrophils and vascular endothelial cells (Camussi, G. *et al., J. Exp. Med. 166*:1390 (1987)). Recent evidence implicates TNF in the pathogenesis of many infections (Cerami, A. *et al., Immunol. Today 9*:28 (1988)), immune disorders, neoplastic pathology, e.g., in cachexia accompanying some malignancies (Oliff, A. *et al., Cell 50*:555 (1987)), and in autoimmune pathologies and graft-versus host pathology (Piguet, P.-F. *et al., J. Exp. Med. 166*:1280 (1987)). A number of studies have suggested that TNF is an important mediator of the cachexia in cancer, infectious pathology, and in other catabolic states.

Thus, the endokine alpha protein of the present invention can be used for tumor targeting, preferably, after conjugation with radioisotypes or cytostatic drugs (Gruss and Dower, *Blood 85(12)*:3378-3404 (1995)). Endokine alpha can be used in patients with melanoma and sarcoma for tumor regression and extension of patient life span through a local injection or used in isolated limb perfusion (Aggarwal and Natarajan, *Eur. Cytokine Netw. 7(2)*:92-124 (1996)).

The endokine alpha of the present invention can also have a therapeutic role in specific situations, for example, activity against viral, bacterial, yeast,

As endokine alpha is believed to exhibit many of the biological effects of TNF, the present invention is further directed antibody-based therapies which involve administering an anti-endokine alpha antibody to a mammalian, preferably human, patient for treating one or more of the above-described disorders. Methods for producing anti-endokine alpha polyclonal and monoclonal antibodies are described in detail above. Such antibodies may be provided in pharmaceutically acceptable compositions as known in the art or as described herein.

A summary of the ways in which the antibodies of the present invention may be used therapeutically includes binding endokine alpha locally or systemically in the body or by direct cytotoxicity of the antibody, *e.g.*, as mediated by complement (CDC) or by effector cells (ADCC). Some of these approaches are described in more detail below. Armed with the teachings provided herein, one of ordinary skill in the art will know how to use the antibodies of the present invention for diagnostic, monitoring or therapeutic purposes without undue experimentation.

The pharmaceutical compositions of the present invention may be administered by any means that achieve their intended purpose. Amounts and regimens for the administration of antibodies, their fragments or derivatives can be determined readily by those with ordinary skill in the clinical art of treating TNF-related disease.

For example, administration may be by parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

Compositions within the scope of this invention include all compositions wherein the antibody, fragment or derivative is contained in an amount effective to achieve its intended purpose. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the

The antibodies of this invention may be advantageously utilized in combination with other monoclonal or chimeric antibodies, or with lymphokines or hemopoietic growth factors, etc., which serve to increase the number or activity of effector cells which interact with the antibodies.

Since circulating concentrations of endokine alpha (like TNF) tend to be extremely low, in the range of about 10 pg/ml in non-septic individuals, and reaching about 50 pg/ml in septic patients and above 100 pg/ml in the sepsis syndrome for TNF (Hammerle, A.F. *et al.*, 1989, *supra*) or may be only be detectable at sites of endokine alpha-related disorders, it is preferred to use high affinity and/or potent *in vivo* endokine alpha-inhibiting and/or neutralizing antibodies, fragments or regions thereof, for both endokine alpha immunoassays and therapy of endokine related disorders. Such antibodies, fragments, or regions, will preferably have an affinity for human endokine alpha, expressed as Ka, of at least 10^8 M^{-1}, more preferably, at least 10^9 M^{-1}, such as 5 X 10^8 M^{-1}, 8 X 10^8 M^{-1}, 2 X 10^9 M^{-1}, 4 X 10^9 M^{-1}, 6 X 10^9 M^{-1}, 8 X 10^9 M^{-1}.

Preferred for human therapeutic use are high affinity murine and murine/human or human/human chimeric antibodies, and fragments, regions and derivatives having potent *in vivo* endokine-inhibiting and/or neutralizing activity, according to the present invention, *e.g.*, that block endokine-induced IL-1, IL-6 or TNF secretion, procoagulant activity, expression of cell adhesion molecules such as ELAM-1 and ICAM-1 and mitogenic activity, *in vivo*, *in situ*, and *in vitro*.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

containing multiple copies of the plasmid pREP4, which expresses lac repressor and confers kanamycin resistance ("Kan"), is used in carrying out the illustrative example described herc. This strain, which is only one of many that are suitable for expressing endokine alpha protein, is available commercially from Qiagen.

Transformants are identified by their ability to grow on LB plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis.

Clones containing the desired constructs are grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 µg/ml) and kanamycin (25 µg/ml).

The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:100 to 1:250. The cells are grown to an optical density at 600 NM ("OD600") of between 0.4 and 0.6. Isopropyl-B-D-thiogalactopyranoside ("IPTG") is then added to a final concentration of 1 mM to induce transcription from *lac* repressor sensitive promoters, by inactivating the *lac*I repressor. Cells subsequently are incubated further for 3 to 4 hours. Cells then are harvested by centrifugation and disrupted, by standard methods. Inclusion bodies are purified from the disrupted cells using routine collection techniques, and protein is solubilized from the inclusion bodies into 8M urea. The 8M urea solution containing the solubilized protein is passed over a PD-10 column in 2X phosphate-buffered saline ("PBS"), thereby removing the urea, exchanging the buffer and refolding the protein. The protein is purified by a further step of chromatography to remove endotoxin. Then, it is sterile filtered. The sterile filtered protein preparation is stored in 2X PBS.

Example 2: Cloning and Expression of Mature Endokine Alpha in a Baculovirus Expression System

The cDNA sequence encoding the entire endokine alpha protein in the deposited clone is amplified using PCR oligonucleotide primers corresponding to 5' and 3' regions of the gene.

a signal peptide, as required. Such vectors are described in Luckow *et al.*, *Virology* 170: 31-39, among others.

The plasmid is digested with the restriction enzyme XbaI and then is dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated herein "V2".

Fragment F2 and the dephosphorylated plasmid V2 are ligated together with T4 DNA ligase. *E. coli* HB101 cells are transformed with ligation mix and spread on culture plates. Bacteria are identified that contain the plasmid with the human endokine alpha gene by digesting DNA from individual colonies using XbaI and then analyzing the digestion product by gel electrophoresis. The sequence of the cloned fragment is confirmed by DNA sequencing.

5 µg of the plasmid is co-transfected with 1.0 µg of a commercially available linearized baculovirus DNA ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.), using the lipofection method described by Felgner et al., Proc. Natl. Acad. Sci. USA 84: 7413-7417 (1987). 1µg of BaculoGold™ virus DNA and 5 µg of the plasmid are mixed in a sterile well of a microtiter plate containing 50 µl of serum-free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10 µl Lipofectin plus 90 µl Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is rocked back and forth to mix the newly added solution. The plate is then incubated for 5 hours at 27°C. After 5 hours the transfection solution is removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. The plate is put back into an incubator and cultivation is continued at 27°C for four days.

After four days the supernatant is collected and a plaque assay is performed, as described by Summers and Smith, cited above. An agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used to allow easy

cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (*see*, e.g., Alt, F.W. *et al. J. Biol. Chem. 253*:1357-1370 (1978), Hamlin, J.L. and Ma, C., *Biochim. et Biophys. Acta, 1097*:107-143 (1990), Page, M.J. and Sydenham, M.A., *Biotechnology 9*:64-68 (1991)). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene it is usually co-amplified and over-expressed. It is state of the art to develop cell lines carrying more than 1,000 copies of the genes. Subsequently, when the methotrexate is withdrawn, cell lines contain the amplified gene integrated into the chromosome(s).

Plasmid pC1 contains for the expression of the gene of interest a strong promoter of the long terminal repeat (LTR) of the Rouse Sarcoma Virus (Cullen, *et al.*, *Molecular and Cellular Biology*, 438-4470 (March 1985)) plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV) (Boshart *et al.*, *Cell 41*:521-530 (1985)). Downstream of the promoter are the following single restriction enzyme cleavage sites that allow the integration of the genes: BamHI, Pvull, and Nrul. Behind these cloning sites the plasmid contains translational stop codons in all three reading frames followed by the 3' intron and the polyadenylation site of the rat preproinsulin gene. Other high efficient promoters can also be used for the expression, e.g., the human β-actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLVI. For the polyadenylation of the mRNA other signals, e.g., from the human growth hormone or globin genes can be used as well.

Stable cell lines carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g. G418 plus methotrexate.

the plasmid pSVneo using the lipofecting method (Felgner *et al.*, *supra*). The plasmid pSV2-neo contains a dominant selectable marker, the gene neo from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) and cultivated from 10-14 days. After this period, single clones are trypsinized and then seeded in 6-well petri dishes using different concentrations of methotrexate (25 nM, 50 nM, 100 nM, 200 nM, 400 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (500 nM, 1 μM, 2 μM, 5 μM). The same procedure is repeated until clones grow at a concentration of 100 μM.

The expression of the desired gene product is analyzed by Western blot analysis and SDS-PAGE.

Example 4: Tissue distribution of Endokine Alpha expression

Northern blot analysis was carried out to examine the levels of expression of the gene encoding the endokine alpha protein in human tissues, using methods described by, among others, Sambrook *et al.*, *supra*. A cDNA probe containing the entire nucleotide sequence of the endokine alpha protein of the present invention (SEQ ID NO:1) was labeled with ^{32}P using the *redi*prime™ DNA labeling system (Amersham Life Science), according to manufacturer's instructions. After labelling, the probe was purified using a CHROMA SPIN-100™ column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labelled probe was then used to examine various human tissues for the expression of the gene encoding the endokine alpha protein.

Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) were obtained from Clontech and were examined with labelled probe using ExpressHyb™ Hybridization Solution (Clontech) according to manufacturer's protocol number PT1190-1. Following

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Human Genome Sciences, Inc.
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Rosen, Craig A.

(ii) TITLE OF INVENTION: Human Endokine Alpha

(iii) NUMBER OF SEQUENCES: 10

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT To be assigned
(B) FILING DATE: Herewith
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1809 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

TCAGCACATG TAGAGGTGCC AGTGGGTGGA TTGGAGGGAG AAGATATTCA ATTTCTAGAG 649

TTTCTGTCTA CAAAAATCAA CACAAACAGA ACTCCTCTGC ACGTGAATTT TCATCTATCA 709

TGCATCTGAA AGAGACTCAG GGGAAAAGCC AAAGACTTTT GGTTGGATCT GCAGAGATAC 769

TTCTAATCCA TGATAAAACA AATATGGATG ACAGAGGACA TGTGCTTTTC AAAGAATCTT 829

TATAATTCTT GAATTCATGA GTGGAAAAAT GGAGTTCTAT TCCCATGGAA GATTTACCTG 889

GTACAAAAAG GATCTGGGGC AGTAGCCTGG CTTTGTTCTC ATATTCTTGG GCTGCTGTAA 949

TTCTCTTCTC ATACTCCCAT CTTCTGAGAC CCTCCCAATA AAAAGTAGAC TGATAGGATG 1009

GCCAGATATG CCTACCATAC CCTACTTTAG ATATGGTGGT GTTAGAAGAT AAAGAACAAT 1069

CTGAACTATT GGAATAGAGG TACAAGTGGC ATAAAATGGA ATGTACGCTA TCTGGAAATT 1129

TCTTGGTTTT ATCTTCCTCA GGATGCAGGG TGCTTTAAAA AGCCTTATCA AAGGAGTCAT 1189

TCCACCCTCA CGTAGAGCTT TGTGAGAACT TACTGTTGGT GTGTGTGTCT AAACATTGCT 1249

AATTAAAGAA AGAGTAACCA TTAGTAATCA TTAGGTTTAA CCCCAGAATG GTATTATCAT 1309

TACGATTATG TCATGTAATG ATTTAGTATT TTTAGCTAGC TTTCCACAGT TTGCAAAGTG 1369

CTTGTAAAAC AGTTAGCAAT TCTATGAAGT TAATTGGGCA GGCATTTGGG GGAAAATTTT 1429

AGTTGAGAAT GTGATAGCAT AGCATAGCCA ACTTTCCTCA ACTCATAGGA CAAGTGACTA 1489

CAAGGCAATG GGTAGTCCCC TGCATTGCAC TGTCTCAGCT TTAGAATTGT TATTTCTGCT 1549

ATCGTTATAA GACTCTAAAA CTTAGCGAAT TCACTTTTCA GGAAGCATAT TCCCCTTTAG 1609

CCCGGTGAGC AGAGTGAAGC TACAACAGAT CTTTCCTTTA CCAGCACACT TTTTTTTTTT 1669

TCCCCTGAAT CAGGGAGATC CAGGATGCTG TTCAGGCCTT ATCCCAACCA AATTCCCCTC 1729

TTCTTTGCAG GGCCCATCTT AGTCAAATGT GCTAACTTCT AAAATAATAA ATAGCACTAA 1789

TTCAAAAAAA AAAAAAAAAA 1809

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 169 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Pro Leu Ser His Ser Arg Thr Gln Gly Ala Gln Arg Ser Ser Trp

Cys Leu Leu His Phe Gly Val Ile Gly Pro Gln Arg Glu Glu Ser Pro
50 55 60

Arg Asp Leu Ser Leu Ile Ser Pro Leu Ala Gln Ala Val Arg Ser Ser
65 70 75 80

Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val Val Ala Asn Pro
85 90 95

Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg Ala Asn Ala Leu
100 105 110

Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu Val Val Pro Ser
115 120 125

Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe Lys Gly Gln Gly
130 135 140

Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile Ser Arg Ile Ala
145 150 155 160

Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala Ile Lys Ser Pro
165 170 175

Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro Trp Tyr Glu
180 185 190

Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu
195 200 205

Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser Gly
210 215 220

Gln Val Tyr Phe Gly Ile Ile Ala Leu
225 230

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 205 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Thr Pro Pro Glu Arg Leu Phe Leu Pro Arg Val Cys Gly Thr Thr
1 5 10 15

Leu His Leu Leu Leu Leu Gly Leu Leu Leu Val Leu Leu Pro Gly Ala

(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGAAGCTTT CAAGTCTCTA GGAGATG 27

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGGATCCCG AGACTGCTAA GGAGCC 26

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCGGATCCCT AGGAGATGAA TTGGGGATTT G 31

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 5, line 20

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*
12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
June 27, 1996	ATCC 97640

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

DNA PLASMID HG944451

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

3. An isolated nucleic acid molecule comprising a polynucleotide which encodes the amino acid sequence of an epitope-bearing portion of an endokine alpha polypeptide having an amino acid sequence in (a), (b), (c), (d), or (e) of claim 1.

4. The isolated nucleic acid molecule of claim 3, which encodes an epitope-bearing portion of an endokine alpha polypeptide selected from the group consisting of: a polypeptide comprising amino acid residues from about 44 to about 158 in FIG. 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 44 to about 54 in FIG. 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 57 to about 68 in FIG. 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 69 to about 78 in FIG. 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 94 to about 105 in FIG. 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 108 to about 132 in FIG. 1 (SEQ ID NO:2); and a polypeptide comprising amino acid residues from about 148 to about 158 in FIG. 1 (SEQ ID NO:2).

5. The isolated nucleic acid molecule of claim 1, wherein said polynucleotide is DNA.

6. The isolated nucleic acid molecule of claim 1, wherein said polynucleotide is RNA.

7. A method for making a recombinant vector comprising inserting an isolated nucleic acid molecule of claim 1 into a vector.

8. A recombinant vector produced by the method of claim 7.

9. A method of making a recombinant host cell comprising introducing the recombinant vector of claim 8 into a host cell.

comprising amino acid residues from about 69 to about 78 in FIG. 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 94 to about 105 in FIG. 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 108 to about 132 in FIG. 1 (SEQ ID NO:2); and a polypeptide comprising amino acid residues from about 148 to about 158 in FIG. 1 (SEQ ID NO:2).

14. An isolated antibody or antibody fragment that binds specifically to an endokine alpha polypeptide of claim 12.

15. A method for treating an individual in need of a decreased level of endokine alpha activity, comprising

administering to said individual a composition comprising the isolated antibody or antibody fragment of claim 14.

16. A diagnostic method, comprising:

(a) assaying endokine alpha gene expression level in mammalian cells or body fluid; and

(b) comparing said endokine alpha gene expression level with a standard endokine alpha gene expression level, whereby an increase or decrease in said endokine alpha gene expression level compared to said standard is indicative of a TNF-related disorder.

GTAAAACAGTTAGCAATTCTATGAAGTTAATTGGGCAGGCATTTGGGGGAAAATTTTAGT
TGAGAATGTGATAGCATAGCATAGCCAACTTTCCTCAACTCATAGGACAAGTGACTACAA
GGCAATGGGTAGTCCCCTGCATTGCACTGTCTCAGCTTTAGAATTGTTATTTCTGCTATC
GTTATAAGACTCTAAAACTTAGCGAATTCACTTTTCAGGAAGCATATTCCCCTTTAGCCC
GGTGAGCAGAGTGAAGCTACAACAGATCTTTCCTTTACCAGCACACTTTTTTTTTTTTCC
CCTGAATCAGGGAGATCCAGGATGCTGTTCAGGCCTTATCCCAACCAAATTCCCCTCTTC
TTTGCAGGGCCCATCTTAGTCAAATGTGCTAACTTCTAAAATAATAAATAGCACTAATTC
AAAAAAAAAAAAAAAAA

FIG.1B

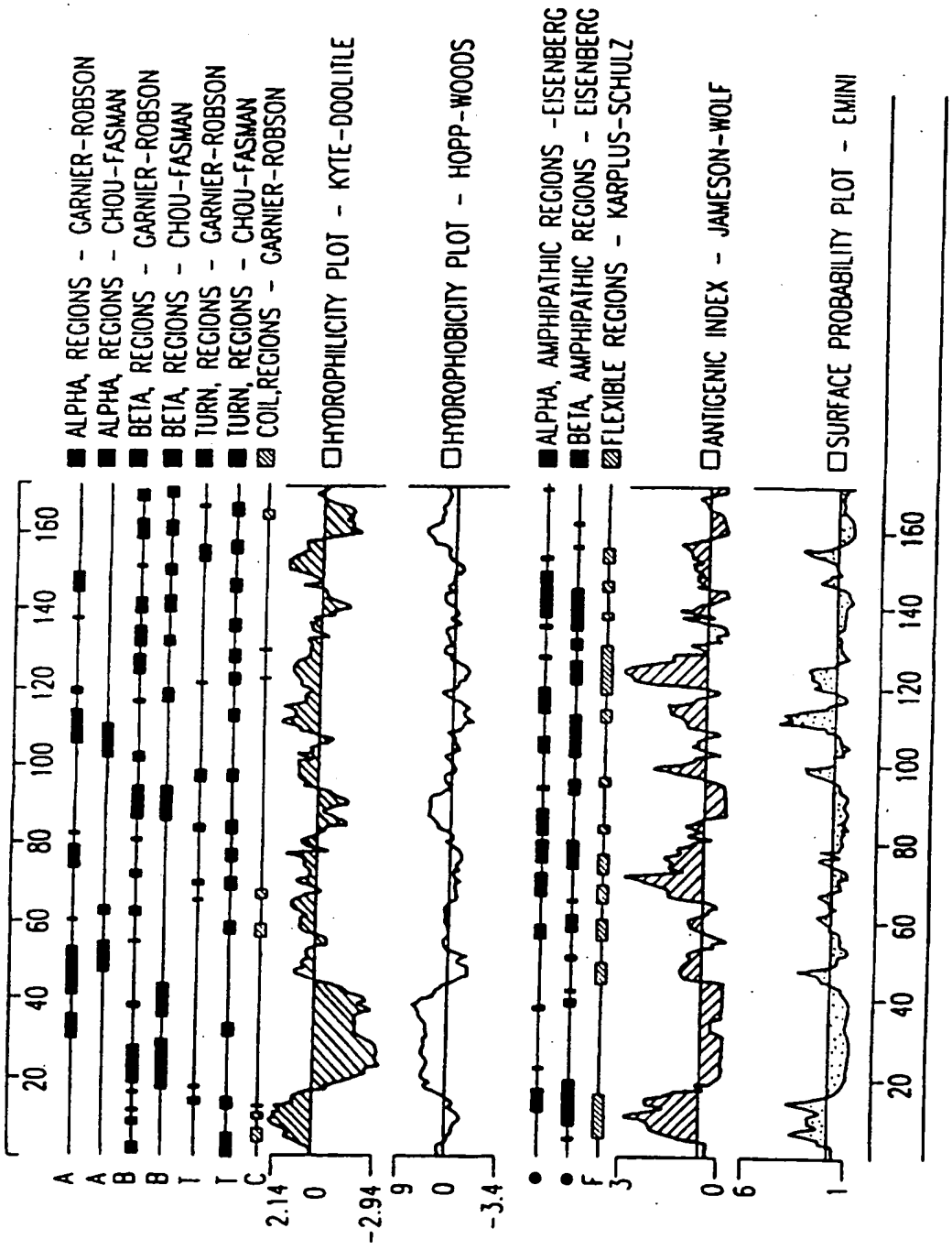
ALPHA, REGIONS - GARNIER-ROBSON
ALPHA, REGIONS - CHOU-FASMAN
BETA, REGIONS - GARNIER-ROBSON
BETA, REGIONS - CHOU-FASMAN
TURN, REGIONS - GARNIER-ROBSON
TURN, REGIONS - CHOU-FASMAN
COIL,REGIONS - GARNIER-ROBSON
HYDROPHILICITY PLOT - KYTE-DOOLITLE
HYDROPHOBICITY PLOT - HOPP-WOODS
ALPHA, AMPHIPATHIC REGIONS -EISENBERG
BETA, AMPHIPATHIC REGIONS - EISENBERG
FLEXIBLE REGIONS - KARPLUS-SCHULZ
ANTIGENIC INDEX - JAMESON-WOLF
SURFACE PROBABILITY PLOT - EMINI
20 40 60 80 100 120 140 160
2.14 0 -2.94
9 0 -3.4
3 0
6 1

FIG.3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/13282

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Database EST-STN on MASPAR search, GenBank at National Library of Medicine, No. S78214, 10 July 1992, MIKI et al., "Disruption of the APC gene by retrotransposal insertion of L1 sequence in a colon cancer", Cancer Research, Vol. 52 No.3, 1992, pages 643-645, see sequence alignment.	1-13
A	EMBL/GENBANK/DDBJ DATA BANKS, No. P41086, EMBL U02603, WOOD, "Putative Succinate Dehydrogenase 15 KD Hydrophobic Protein:", 01 February 1995, see sequence alignment.	1-13